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Modulation of gene expression after replication-deficient, recombinant adenovirus-mediated gene transfer by the product of a second adenovirus vector

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To regulate gene expression following adenovirus-mediated gene transfer, a strategy was devised utilizing co-infection with two separate adenovirus vectors designed such that the product of one vector modulated the promoter of the second vector. To evaluate this strategy, AdEGR1.TNF, an adenovirus expressing tumor necrosis factor-α (TNF) under the control of the early growth response 1 (EGR1) promoter, was used to regulate a transcription unit in AdIL8.βgal, an adenovirus vector in which the TNF sensitive interleukin-8 (IL-8) promoter drives the expression of β-galactosidase (β-gal). Following infection of HS24 cells with AdIL8.βgal, addition of TNF to the culture induced the expression of β-gal. Infection of HS24 cells with AdEGR1.TNF resulted in a dose-dependent secretion of TNF. Little β-gal was produced following co-infection of the cells with the control vector AdCMV.Null (expressing no specific gene) and AdIL8.βgal. In contrast, co-infection with AdIL8.βgal and AdEGR1.TNF demonstrated, for a given dose of AdIL8.βgal, increasing amounts of β-gal expression dependent on the dose of AdEGR1.TNF. This model suggests control of gene expression in adenovirus-mediated gene transfer can be regulated by utilizing a promoter-gene expression cassette in one vector that modulates the expression of a promoter-gene expression cassette in a second vector.

Keywords: gene therapy; adenovirus; promoter; interleukin-8; tumor necrosis factor

Introduction

Replication deficient, recombinant adenovirus (Ad) vectors are highly efficient at transferring genes *in vitro* and *in vivo*, and are being used in a wide variety of applications in cell culture, experimental animals and human gene therapy.[1–14] For most of these applications, the gene to be expressed has been controlled by constitutive promoters, such as the Ad type 2 or 5 major late promoter and tripartite leader, the cytomegalovirus (CMV) immediate–early promoter/enhancer, the Rous sarcoma virus long terminal repeat and others.[1–14] To a varying degree all of these promoters are successful in achieving the desired expression of the exogenous gene.

The use of constitutive promoters, however, is limited by the lack of control once the gene is transferred. While this may not be a problem for some applications, such as gene therapy for the respiratory manifestations of cystic fibrosis,[2,4,5,15] it is not acceptable in circumstances where the product of the gene to be transferred is potentially toxic or where physiologic regulation is required. For such applications, an ideal expression cassette to be transferred would include a promoter that could be up- or down-regulated in response to appropriate signals. One obvious strategy to maintain control of the transferred gene is to transfer an expression cassette that includes a promoter that can be regulated, rather than a constitutive promoter. One problem with this approach is that most promoters that can be regulated are responsive to a variety of stimuli, and thus the expression cassette may be turned on or off inappropriately.

An alternative method of regulating gene expression in adenovirus-mediated gene therapy, is to use two separate replication deficient recombinant vectors, with the promoter of the 'therapeutic' gene in vector 1 controlled by the product of the expression cassette in vector 2. To evaluate this concept, the replication-deficient adenovirus vectors AdIL8.βgal and AdEGR1.TNF were constructed. AdIL8.βgal contains the *Escherichia coli lacZ* reporter gene [coding for β-galactosidase (β–gal)] controlled by the 5' flanking region of the human interleukin-8 (IL-8) gene, a promoter known to contain elements responsive to tumor necrosis factor-α (TNF).[16,17] The second vector (AdEGR1.TNF), was used to direct the production of TNF under the control of the basal activity of the early growth response 1 (EGR1) promoter [comprising bp −425 to +65 of the 5' flanking region of the murine EGR1 gene].[18,19] The data demonstrate that signals generated by one adenovirus vector can be used to modulate gene expression directed by the expression cassette transferred to the infected cell population by a

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second vector, a strategy that has a number of possible gene therapy applications.

Results

Modulation of β-galactosidase activity by TNF after infection with AdIL8.βgal

As expected, the expression cassette comprising the IL-8 promoter and the β–gal gene transferred to cells by the vector AdIL8.βgal was responsive to exogenously added TNF, with the AdIL8.βgal vector directing increasing amounts of β–gal expression in response to TNF (Figure 1). In this regard, uninfected HS24 cells expressed no β–gal (Figure 1A). Uninfected cells exposed to TNF also showed no β–gal expression (Figure 1B). In contrast, 72 h after

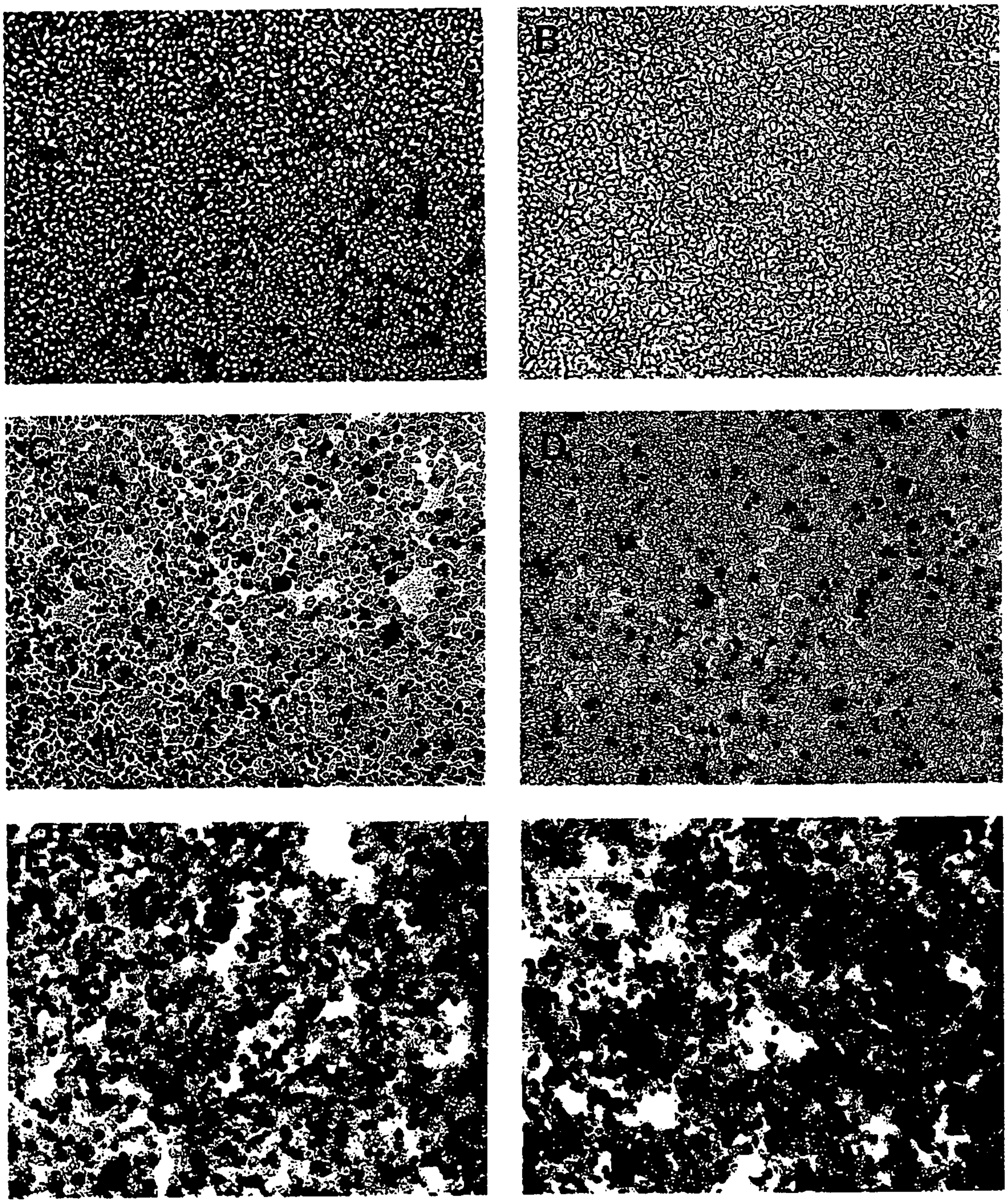

Figure 1 Modulation of β-galactosidase activity in HS24 cells with exogenous TNF following infection with AdIL8.βgal. Cells were infected with the AdIL8.βgal vector at a multiplicity of infection of 25 or 100 plaque forming units (p.f.u.)/cell and then exposed to increasing amounts of TNF. (A) Uninfected cells. (B) Uninfected cells exposed to TNF (10^4 U/ml). (C) Cells infected with 100 p.f.u./cell AdIL8.βgal, not exposed to TNF. (D) Cells infected with 25 p.f.u./cell AdIL8.βgal, exposed to TNF (10^4 U/ml). (E) Cells infected with 100 p.f.u./cell AdIL8.βgal, exposed to TNF (10^3 U/ml). (F) Cells infected with 100 p.f.u./cell AdIL8.βgal and exposed to TNF (10^4 U/ml). β-Galactosidase activity is demonstrated by a blue color using the X-gal reagent. (A–F) Original magnification ×110

infection with AdIL8.βgal, there were increasing amounts of β–gal expression dependent on the dose of AdIL8.βgal and TNF (Figure 1C–F). Quantitative assessment of β–gal activity in the cell lysates demonstrated that the up-regulation of β–gal activity was dependent on two parameters: the multiplicity of infection with the AdIL8.βgal vector and the amount of TNF added to the cultures (Figure 2). In the absence of TNF, the level of β–gal activity increased slightly with the dose of the virus. TNF did not significantly affect baseline β–gal activity in uninfected cells. However, if cells were infected with AdIL8.βgal and TNF was added at an increasing dose, β–gal expression increased markedly, with a 10-fold increase from baseline at the highest doses of vector and TNF.

TNF secretion after AdEGR1.TNF-mediated gene transfer

Uninfected HS24 cells or HS24 cells infected with AdIL8.βgal or AdCMV.Null resulted in no detectable TNF in the culture supernatant (Figure 3). In contrast, after infection with AdEGR1.TNF, TNF was easily detected in the supernatants with the amount of TNF correlating with the dose of the vector.

Modulation of β-galactosidase activity by co-infection with AdIL8.βgal and AdEGR1.TNF

Uninfected HS24 cells, cells infected with AdIL8.βgal alone, or cells infected with AdEGR1.TNF alone generated little or no β–gal activity (Figure 4A–C). In contrast, when cells were infected with the two vectors, β–gal activity was clearly evident, with increasing expression dependent on the amount of the AdEGR1.TNF vector (Figure 4D–F).

Quantitative assessment of β–gal activity in the HS24 cell lysates infected with increasing amounts of the control vector AdCMV.Null alone demonstrated

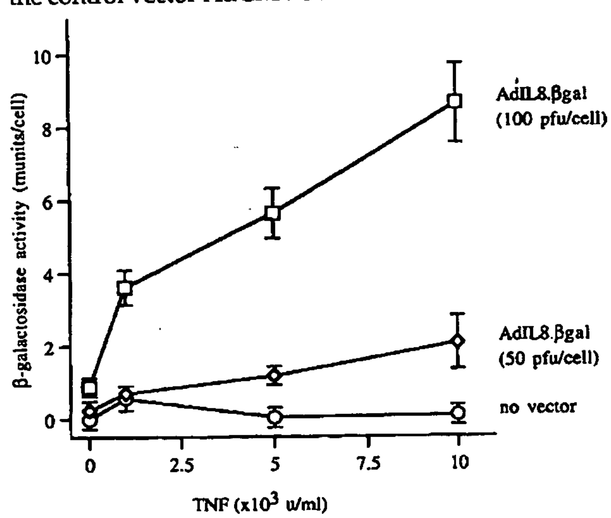

Figure 2 Quantitative measurement of β-galactosidase activity in HS24 cells associated with the addition of TNF following infection with AdIL8.βgal. The cells were infected with the AdIL8.βgal vector at a MOI of 50 or 100 p.f.u./cell and then exposed to increasing amounts of TNF. β-Galactosidase activity was quantified in cell lysates (mU/cell) 72 h after infection. The data represent an average of triplicate determinations

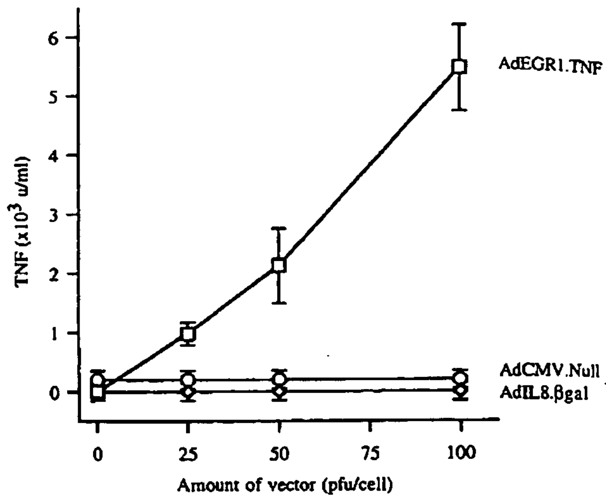

Figure 3 Secretion of TNF from HS24 cells after infection with AdEGR1.TNF. The cells were infected with increasing amounts of AdEGR1.TNF and after 72 h, the supernatants were evaluated for TNF activity (μ/ml). The vectors AdCMV.Null and AdIL8.βgal are used as controls. The data represent an average of triplicate determinations

no induction of β–gal expression (Figure 5A). The same was true for HS24 cells co-infected with AdCMV.Null and AdIL8.βgal, except at the highest doses of both vectors where low-level β–gal activity was detected. In marked contrast, while infection with increasing amounts of the AdEGR1.TNF vector was associated with no detectable β–gal expression, co-infection with AdEGR1.TNF and AdIL8.βgal showed dose-dependent induction of β–gal expression, with dose-dependent increase of β–gal activity for both vectors (Figure 5B). With the highest dose of the AdEGR1.TNF vector [100 plaque forming units (p.f.u.)/cell], at the highest dose of the AdIL8.βgal vector (100 p.f.u./cell) there was a five-fold increase in β–gal activity over AdIL8.βgal vector alone; a level significantly greater than that observed with co-infection at the same doses of the control vector AdCMV.Null and the AdIL8.βgal vector (two-tailed Student's t test; $P<0.005$).

Discussion

One approach to control of gene expression in gene therapy models is to utilize a promoter that will respond either directly or indirectly to various stimuli.[17,20–22] As an extension of this concept, a control strategy was developed using two recombinant adenovirus vectors, designed such that the gene product of one vector modulates the expression of the gene product of the second vector. As a model to evaluate this strategy, the present study demonstrates that co-infection of a lung cell line with an adenovirus vector constitutively expressing TNF will activate a TNF-responsive promoter in a second vector to, in turn, activate a reporter gene. There are several possible applications of such a control system for gene therapy.

Enhancing target specificity

First, a co-infection strategy with two interdependent vectors could provide improvement in target cell

specificity for *in vivo* gene therapy, without relying on tissue-specific promoters. Although experimental animal studies will be necessary to prove such interdependence will be observed *in vivo*, all available evidence suggests such studies will work as proposed. A two-vector co-infection strategy confers specificity because: (1) the two vectors are constructed to be interdependent, eg the product of vector 1 activates the promoter of vector 2; and (2) the likelihood of a cell being infected by two different vectors is the square of the probability that a cell will be infected by one vector.[23] For example, intravenous administration of an adenovirus vector with a constitutive viral promoter controlling a reporter gene into mice results in >90% chance that hepatocytes express the reporter gene, with <1% chance that cells

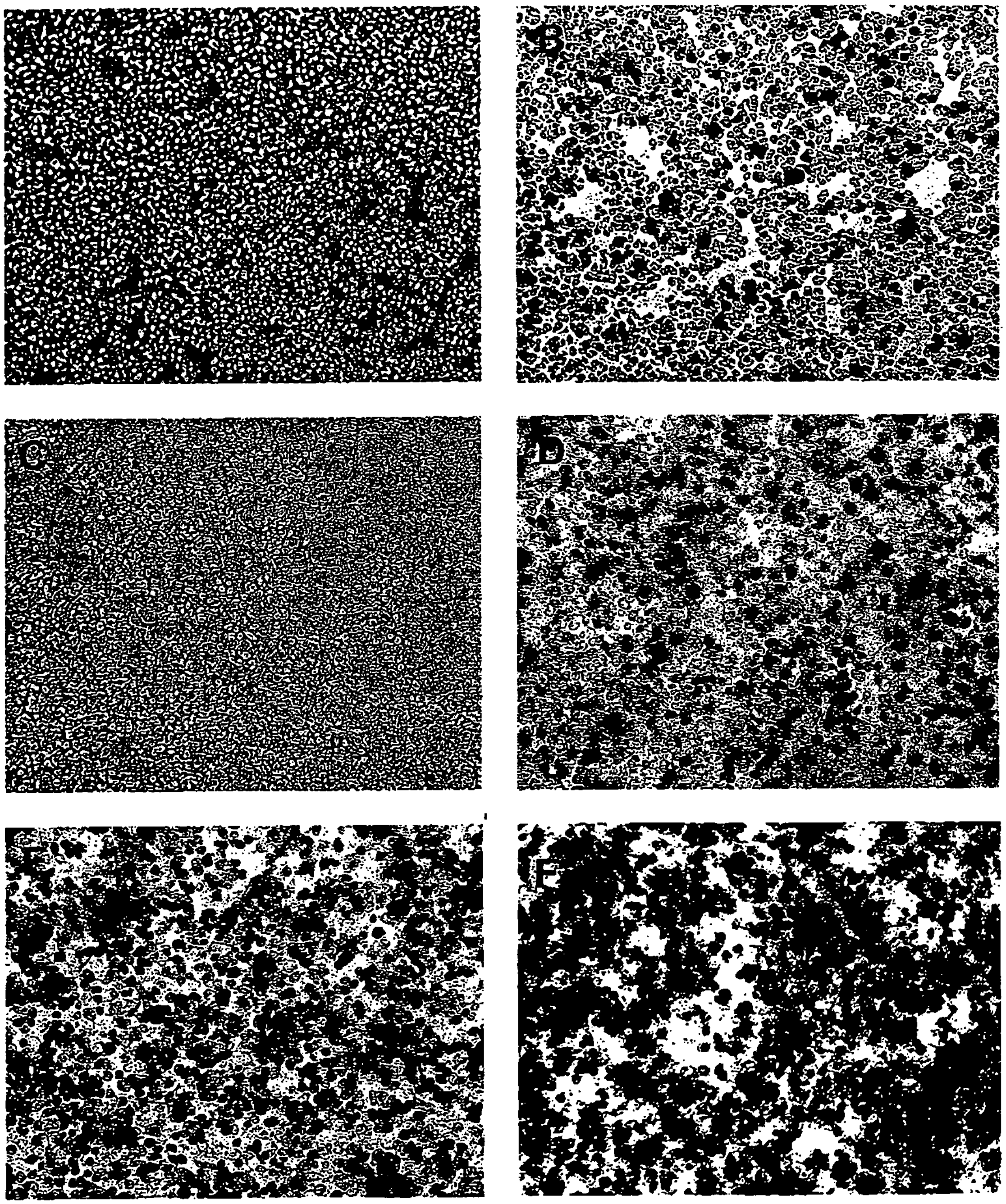

Figure 4 Expression of β-galactosidase in HS24 cells after co-infection with AdIL8.βgal and AdEGR1.TNF. The cells were infected with AdIL8.βgal at a MOI of 100 p.f.u./cell and increasing amounts of AdEGR1.TNF. β-Galactosidase activity is indicated by a blue color as evaluated by the X-gal assay. (A) Uninfected cells. (B) Cells infected only with AdIL8.βgal (100 p.f.u./cell). (C) Cells infected only with AdEGR1.TNF (100 p.f.u./cell). (D) Cells infected with AdIL8.βgal (25 p.f.u./cell) and AdEGR1.TNF (100 p.f.u./cell). (E) Cells infected with AdIL8.βgal (100 p.f.u./cell) and AdEGR1.TNF (25 p.f.u./cell). (F) Cells infected with AdIL8.βgal (100 p.f.u./cell) and AdEGR1.TNF (100 p.f.u./cell). (A–F) Original magnification ×110

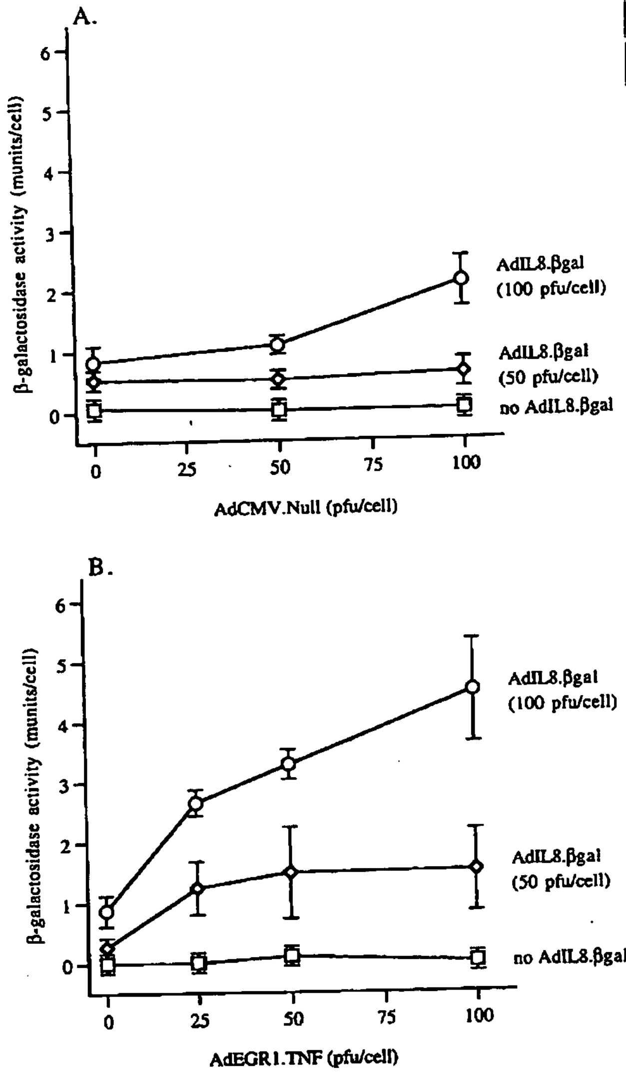

Figure 5 Modulation of β-galactosidase activity in HS24 cells following co-infection of AdIL8.βgal and AdEGR1.TNF. (A) The effect of the control vector AdCMV.Null on the expression of β-galactosidase as directed by AdIL8.βgal. β-Galactosidase activity was determined in HS24 cells following co-infection with AdCMV.Null (0–100 p.f.u./cell) and AdIL8.βgal (0–100 p.f.u./cell). After 72 h β-galactosidase activity was measured in cell lysates (mU/cell). (B) Effect of AdEGR1.TNF on β-galactosidase expression as directed by AdIL8.βgal. The cells were infected with AdIL8.βgal (0–100 p.f.u./cell) and AdEGR1.TNF (0–100 p.f.u./cell) and after 72 h β-galactosidase activity was quantified in the cell lysates (mU/cell). The data represent an average of triplicate determinations

of any other tissues express the gene.[6] Theoretically, if two vectors were administered intravenously, and assuming that, at a given dose of virus, 90% of hepatocytes but only 1% of cells from any other tissue express the gene, there would be an 81% chance that a hepatocyte would express the genes coded by both vectors (90% × 90% = 81%), but only a 0.01% chance (1% × 1% = 0.01%) that a cell in the other tissue would express the gene product of both vectors. Thus, such a co-infection strategy markedly increases selectivity of expression, with a single intravenous administration yielding a 90:1 selectivity ratio of expression in hepatocytes over the other tissue, but with a co-infection strategy with interdependent vectors yielding a selectivity of expression in hepatocytes of 8100:1 over the other tissue.

As another example, direct respiratory tract administration of an adenovirus vector results in marked expression in the respiratory epithelium of the gene carried by the vector, with no detectable expression outside of the lung.[24] If we assume that 25% of the respiratory epithelium will express the new gene, and there was <0.1% chance of any other tissue outside of the lung expressing the gene carried by the vector, a single vector would yield a selectivity of 250:1 of lung epithelium over non-lung tissue (25%/0.1%). In contrast, co-infection of interdependent vectors carrying two different genes would yield a selectivity of expression of both genes of 62 500:1 (25% × 25%/0.1% × 0.1%) for the epithelium compared with any other tissue. Such selectivity could be remarkably useful if the therapeutic product of the second vector was highly toxic, as in cancer therapy applications.

Theoretically, the 'tightness' of the target cell specificity could be enhanced by substituting a tissue-specific promoter in vector 1. For example, if an active, hepatocyte-specific promoter, such as that for albumin or α1-antitrypsin,[25,26] were substituted for the EGR1 promoter used in the model system in the present study, it is likely that the specificity ratio for expression of the model therapeutic gene (β–gal) in hepatocytes over other cells would be even higher than that achieved with the EGR1.TNF and IL8.βgal expression cassettes. Likewise, for tumor applications, the promoter in vector 1 could be a tumor-specific promoter such as the carcinoembryonic antigen for colon carcinoma.[27] Even higher ratios of selectivity could be achieved by utilizing three vectors (or more), with little increase in total adenovirus dose.

Modulation of expression

Second, a two-vector strategy with interdependent vectors may be useful in gene therapy applications in which the potential for induction (or suppression) of the expression of the therapeutic gene is desired. One approach might be to use the second of the two vectors intermittently as the inducing or suppressing stimulus for the promoter-therapeutic gene expression cassette previously administered in the first vector. For example, using the two model vectors in the present study, if the AdIL8.βgal vector was administered to an organ first, the infected cells would not be exposed to much β–gal (the model therapeutic gene), since the IL8 promoter has little constitutive activity.[17] At a later time, when the activity of the therapeutic gene was desired, the second vector containing the 'on-switch' for the promoter of the previously administered vector could be given, ie administration of the AdEGR1.TNF vector would result in activation of the IL8 promoter in the previously administered AdIL8.βgal expression cassette. Using this concept, a variety of strategies might be envisioned, where the promoter of the first vector contained multiple 'on' or 'off' switches, and

the second vector (or multiple other vectors) would contain the stimuli to up- or down-regulate the first vector. In such a scenario the second vector(s) could be administered intermittently to enhance or suppress the activity of the therapeutic gene.

Interleukin-8 promoter

While the observations in the present study support the concept of a co-infection strategy, it is likely that other promoters would be more effective than the IL8 promoter–TNF combination, or that the IL8 promoter could be modified, resulting in improvement in the ratio of the baseline expression to that of the amplified signal secondary to the product of the second vector. Interestingly, in addition to possible uses of the IL8 promoter in a co-infection interdependent vector control strategy, the observations in the present study suggest that the 5'-flanking region of the IL8 gene might be a useful promoter for gene therapy applications involving epithelial cells (and possibly other cell types). In this regard, transfer of the IL8.βgal expression cassette demonstrated that the IL8 promoter is responsive to TNF in a human lung carcinoma cell line. Since tumors are often accompanied by local inflammation, or local inflammation could be induced in tumors by various stimuli, the IL8 promoter might be useful in gene therapy applications to regulate a therapeutic gene using the local inflammation to up-regulate the expression of the gene. Such a strategy might also be used in non-cancer applications, including the use of anti-inflammatory therapeutic genes, where the IL8 promoter would sense local inflammation, and up-regulate the therapeutic gene which would, in turn, suppress inflammation.

Materials and methods

Vector construction

AdEGR1.TNF, an $E1a^-$, partial $E1b^-$, partial $E3^-$ Ad5 based adenovirus vector, which contained an expression cassette replacing the E1 region consisting of the mouse early growth response gene 1 (EGR1) promoter, SV40 splice signal, human TNF cDNA and SV40 polyadenylation signal, was constructed by homologous recombination in 293 cells by transfection of the plasmids pEGR1.TNF [containing 452 bp of the left end of Ad5, 490 bp (from −425 to +65) of the mouse EGR1 5'-flanking region,[19] 180 bp of the SV40 splice signal,[28] the TNF cDNA,[29] and 196 bp of SV40 polyadenylation signal,[30] followed by bp 3328 to 5778 of Ad5 as a homologous recombination sequence] and pJM17 (containing the sequence of the Ad5 mutant dl309 and an additional bacterial origin of replication and antibiotic resistance genes).[31] AdIL8.βgal, a similar vector containing an expression cassette including the 379 bp 5'-flanking region of the human IL-8 gene,[17] followed by the *E.coli lacZ* [β-galactosidase (β-gal)] gene,[32] and the SV40 polyadenylation signal, was constructed in a similar fashion. As a control the vector AdCMV.Null was constructed in a similar fashion, but with a cassette containing the enhancer/promoter of the immediate–early gene of the CMV,[33] followed by the SV40 polyadenylation signal (ie a promoter and a polyadenylation signal with no gene to be expressed). As a 'positive control' to insure that the detection system for β–gal was functioning, parallel studies were carried out using AdCMV.NLSβgal, an adenovirus vector similar in design, but with the CMV promoter, a nuclear localization signal, and β–gal as the reporter gene. In all circumstances, the AdCMV.NLSβgal vector yielded β–gal expression in the HS24 cells at parallel doses of the vector. All vectors were plaque purified and amplified in 293 cells, purified and stored as previously described.[2] The overall structure of all vectors was confirmed by polymerase chain reaction (PCR) amplification using specific primers relevant to the expression cassette.

Cell culture

HS24 cells, a human bronchial squamous carcinoma cell line (provided by W. Ebert, Thoraxklinikum, Heidelberg-Rohrbach, Germany) were maintained in 'medium/FCS' [RPMI Medium 1640 (GIBCO BRL, Life Technologies, Grand Island, NY, USA) with 10% fetal calf serum (FCS)], 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO BRL). All studies were carried out in 96-well plates (Falcon 3077, VWR Scientific, Piscataway, NJ). The cells were seeded at a density of 2.5×10^3 cells per well in 100 μl of the medium/FCS. After 24 h, the medium/FCS was removed and replaced with 20 μl of the medium (without FCS) containing one or two of the adenovirus vectors at a multiplicity of infection (MOI) of 0 to 100 each. After 1 h, 100 μl of the medium/FCS was added, and the incubation continued for an additional 72 h. The cultures were then assessed for gene expression and cell number (see below). To evaluate the response of the vectors to exogenous TNF added to the cultures, TNF (lot B31024, 0–10^4 U/ml of supernatant; Genzyme, Boston, MA, USA) was added in 100 μl of the medium/FCS following the 1-h incubation with the vectors as described above, and gene expression was evaluated 72 h later.

Evaluation of recombinant gene expression

To evaluate β–gal expression at the histologic level the cells were washed with phosphate-buffered saline (PBS) pH 7.4 , fixed with 2% formaldehyde in PBS (4°C, 5 min), washed again and then incubated with X-gal substrate solution [1 mg/ml X-gal reagent, (GIBCO BRL), 1.3 mM $MgCl$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ in PBS] at 37°C for 12 h. β-Gal activity was quantified using *o*-nitrophenyl β-D-galactopyranoside as substrate (*o*NPG, Sigma Chemical, St. Louis, MO, USA).[34] Cells were lysed in Hanks' balanced salt solution (HBSS) by five freeze–thaw cycles of the entire 96-well plate, with brief mixing between each cycle. The *o*NPG substrate solution (10 μl of 4 mg/ml of *o*NPG in a buffer consisting of 20 mM NaH_2PO_4, 0.1 mM $MnCl_2$, 2 mM $MgSO_4$, 40 mM 2-mercaptoethanol, pH 7.3) was then added to each well. The reaction was stopped with 50 μl of 1 mM Na_2CO_3. Absorption

was then measured at 405 nm and relative activity calculated [1 unit = $(380 \times A_{405})$/(reaction time in min)].[35]

TNF was assessed in the supernatants (50 µl) of each well by enzyme-linked immunosorbent assay (ELISA; h-Tumor Necrosis Factor-α-Elisa kit, Boehringer Mannheim Biochemica, Indianapolis, IN, USA) following the protocol provided by the manufacturer.

The number of viable cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as substrate in a colorimetric assay [Colorimetric (MTT) Assay; Chemicon International, Temecula, CA, USA]. Cells were counted and a cell number calibration curve constructed for the HS24 cell line. The MTT assay correlated well with cell number for conditions used in this set of experiments. The cell number was not influenced significantly by the experimental procedure (handling, infection or adding TNF). Quantitative measures of β-gal activity are reported as β-gal activity measured per cell (mU/cell), where cell number was taken from the MTT calibration curves from parallel treated wells.

Statistical evaluation

All data are presented as mean ± standard error of the mean (s.e.m.). Comparison between groups was carried out using the two-tailed Student's *t* test.

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